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In vitro Screening of *Lactobacillus* species from Homemade Yoghurt for Antagonistic Effects against Common Bacterial Pathogens

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Abstract

Four species of lactic acid bacteria (LAB) were isolated from homemade yoghurt samples and defined as *Lactobacillus acidophilus Lactobacillus bulgaricus, Lactobacillus casei*, and *Lactobacillus plantarum.* Each LAB isolate was tested for its tolerance to acidic environments at pH (7.0, 4.0, and 2.0). All lactobacilli isolated tolerated acid while *L. bulgaricus* was sensitive to pH 2.0. All four bacteria were resistant to ciprofloxacin. Isolates were further tested for their antimicrobial activity against common pathogenic bacteria such as *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus* (MRSA), and *Bacillus subtilis* using the agar spot method. These lactic acid bacteria were found to inhibit growth of most pathogens tested. The viability of lactobacilli isolates were not affected by storage (for 6 weeks) at -20^{o}C and 4^{o}C but declined when they were stored at room temperature. Acid tolerance and bacterial antagonistic characteristics of these LAB isolates render them good candidates for consideration as probiotics.

Keywords: *Lactobacillus* species, Homemade Yoghurt, Antagonistic Effect, Pathogenic Bacteria, Commercial Probiotics.

1. Introduction

The rapid emergence of drug resistant strains of microbial pathogens, especially those with multiple drug resistances, is a major health problem because of their high occurrence worldwide (Rouveix, 2007).

Infectious diseases are the biggest problem in man. Every year gastrointestinal infections lead to significant morbidity and mortality worldwide (Culligan *et al*., 2009). The World Health Organization (WHO, 2004) estimates that more than 4 billion episodes of diarrhoeal disease occur annually, and that 2.2 million deaths were attributable to enteric infection, making it the fifth leading cause of death at all ages worldwide. Enteric bacteria comprised of *Salmonella* species, *Shigella* sp., *Proteus* sp., *Klebsiella* sp., *E. coli*, *Pseudomonas* sp., *Vibrio cholerae* and *Staphylococcus aureus*, are major etiologic agents of enteric infection (Ballal and Shivananda, 2002). The rise in antibiotic resistant bacteria has awakened the scientific community to the prophylactic and therapeutic uses of probiotics, and to reconsider them as alternatives to antibiotics (Ahmed, 2003).

Consumption of food containing live bacteria is the oldest and still most widely used way to increase the number of advantageous bacteria called "probiotics" in the intestinal tract (Salminen and Von Wright, 2011). Noteworthy, there are large number of probiotic foods that date back to ancient times. These mostly originated from fermented foods, as well as, cultured milk products (Tadesse *et al*., 2005; Kent and Hayward, 2007; Salminen and Von Wright, 2011). The quest to find food ingredients with valuable bioactive properties has created interest in lactic acid bacteria (LAB) with probiotic attributes such as antimicrobial activity against pathogenic microorganisms (Hugo *et al.*, 2006), antiviral activity (Botik, 2007), anti-yeast activity (Kantachote, 2008), and antimutagenic activity (Sung *et al.*, 2006). In addition, probiotic microorganisms have been used as a food preservative and antimicrobial agent more than other chemical agents due to the probiotic effects on human and other animal foods (Hassan *et al.*, 2013). LAB are the most prominent non-pathogenic bacteria that play a vital role in our everyday life, from fermentation, preservation, and production of wholesome foods, and vitamins, to prevention of certain diseases and cancer due to their antimicrobial action (Keith, 1991).

The majority of microorganisms used as probiotics belong to the LAB and bifidobacteria. Within the group of LAB, lactobacilli species are the most commonly utilized group of microorganisms due to their potential beneficiary properties as probiotics. The antagonistic activities of these bacteria are known to inhibit a large number of enteric and urinary pathogenic bacteria (Gilliland, 1990; Battcock and Azam-Ali, 1998; Hutt *et al*., 2006).

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The use of probiotics to control certain infections has started gaining acceptance. The alarming rise of inappropriate antibiotic use, and antimicrobial resistance, along with renewed interest in ecological natural methods to prevent infections, make probiotics a very interesting field for research.

Although, there is a lot of research about antimicrobial activity and antibiotic resistance of LAB, little research was dedicated towards the isolation of LAB from homemade yoghurts. Therefore, the aim of this study was to investigate the antimicrobial activity and antibiotic susceptibility of bacteria isolated from yoghurt samples collected from several regions of Erbil city. These yoghurt bacteria were identified and tested for their resistance to acidic environments, their resistances towards some antibiotics, their antimicrobial activities against Gram-positive and Gram-negative food pathogenic and spoilage microorganisms, and their viability when stored at different temperatures.

2. Materials and Methods

2.1. Sample Collection

Due to its wide acceptance among the consumers of Erbil, homemade dairy yoghurt (known as Erbil yoghurt) was selected for isolation of lactobacilli species. The antagonistic effectiveness of these isolates was compared with commercially available probiotic capsules (Probiotane, Vitane Pharme Germany).

2.2. Isolation of Lactobacilli species from Homemade Yoghurt

A 25 g sample of homemade yoghurt was taken aseptically and homogenized in 225 ml of sterile buffered peptone water. Five 10 fold dilutions of the homogenates were then prepared and were inoculated on plates of de Man Rogosa Sharpe agar (MRS, Oxoid, England), a selective growth medium acidified with 1 N HCl to pH 5.3, and incubated anaerobically using a gas pack (Oxoid, England) for 72 hrs at 37^{o}C. Morphologically distinct and well isolated colonies were subcultured on new MRS agar plates. Finally, pure colonies were obtained.

2.3. Identification of Isolates

Identification of the lactobacilli sp. was performed according to their morphological, cultural, physiological and biochemical characteristics. Macroscopic appearance of all the colonies was examined for cultural and morphological characteristics. Size, shape, color and texture of the colonies were recorded. Isolates were characterized based on Gram's stain reaction, cell morphology, presence of capsule or endospore, motility, catalase reaction, oxidase reaction and by growth at 15^{o}C and 45^{o}C as described by Benson (1994). Tests of, nitrate reduction, sulfide, and indole production, and CO_2 from glucose and H_2S production were performed according to (Merck, 1997). Putative lactobacilli were identified to species level based on the sugar fermentation pattern of the API 20A System (bioMe´ rieux).

2.4. Tolerance to Acidic pH

Tolerance of isolated lactobacilli to acidic pH was determined by growing bacteria in acidic MRS broth. MRS broth was poured in test tubes and pH values 7.0, 4.0 and 2.0 were obtained by addition of 1M HCl or 0.5M NaOH. An amount of 5 log10 CFUs (10^5 CFUs) of each isolated lactobacilli species was inoculated in each broth tube. Test tubes were incubated at 37^{o}C for 120 minutes. Survival of lactobacilli was evaluated by a plate count method (Awan and Rahman, 2005).

2.5. Determination of Antibiotic Susceptibility of the Isolates

Six commonly used antibiotics were used to determine the antibiotic susceptibility of the isolated lactobacilli species using Kirby Bauer method. The antibiotic discs (Oxoid, England) were as follows: Amoxiclav (30µg), Tetracycline (30µg), Vancomycin (30µg), Sulfamethoxazole (100µg), Cefotaxime (10µg) and Ciprofloxacin (5µg).

2.6. Test Pathogens

Pathogenic bacterial isolates were obtained from patients associated with different infections at Rizgary and Komary Hospitals in Erbil city. These isolates were comprised of Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, and Pseudomonas aeruginosa)*, and Gram-positive bacteria (*Staphylococcus aureus (*MRSA) and, *Bacillus subtilis*). Organisms were maintained in Nutrient Agar slants and tubes at 4^{o}C.

2.7. Testing for Antibacterial Activity

Antimicrobial action of all isolated lactobacilli species and commercial probiotics against indicator pathogenic bacteria was determined using the agar spot method. The isolated LAB species and commercial probiotics were grown in MRS broth at 37^{o}C for 48-72 hrs and diluted to a density of 10^8 CFU/ml. Two microliters of each LAB species or commercial probiotics were spotted aseptically onto the MRS agar medium (which was found to support the growth of all the LAB species). The plates were then incubated anaerobically for 48-72 hrs. On the other hand, cultures of the pathogenic bacteria were prepared in nutrient broth and incubated at 37^{o}C for 16-18 hrs. They were then transferred to fresh nutrient broth and incubated at 37^{o}C for 4 hrs. The pathogenic bacterial isolates were then adjusted to 10^8 CFU/ml. One ml of each of the pathogenic bacterial isolates was then inoculated into a flask with 100 ml molten nutrient agar at 50^{o}C. Approximately 7 ml of the agar were poured as an overlay on the plate with the grown LAB, prepared as described above. The former was allowed to solidify, before being incubated aerobically at 37^{o}C for 16-18 hrs. The diameters of the clear zones (in the top agar layer) were measured to the nearest mm. The zones of inhibition were then swabbed, and the swabs were used to inoculate tubes of nutrient broth. After incubation at 37^{o}C for 24 hrs, the tubes were checked for turbidity. A clear tube indicated bactericidal activity of the LAB species, while a turbid tube indicated bacteriostatic (Chuayana *et al.*, 2003). Each assay was done in triplicate.

2.8. Bacterial Viability During Storage

The storage viability of isolated LAB species was recorded weekly at -20^{o}C, 4^{o}C and at room temperature. The test tubes were inoculated with 10^5 CFUs of each

culture suspension. The inoculated test tubes were stored at -20°C, 4°C and room temperature for 6 weeks. The growth was monitored weekly using plate count method in MRS medium.

2.9. Statistical Analysis

All data are expressed as means of standard error (M±SE). Statistical analysis was carried out using SPSS Version 16 software. Data analysis was made using one-way analysis of variance (ANOVA). The comparisons between groups were done using Duncan post hoc analysis. *P* values less than 0.05 were considered statistically significant.

3. Results and Discussion

Increased focus has been given to food as a potential vehicle of antimicrobial substances. Such foods have become an important health care sector in most countries. Among them, are the fermented dairy products and, especially yoghurt, which are classic examples of traditional foods originating from the Middle East and Eastern Mediterranean (Tamime *et al*., 2006; Kyriacou *et al.*, 2008).

In the present study, *Lactobacillus* sp. were isolated from homemade yoghurt common to Erbil city and that is prepared from conventional milk. Based on Gram staining and various biochemical tests, four species of *Lactobacillus* were isolated, namely *L. acidophilus, L. bulgaricus, L. casei and L. plantarum.* Lactic acid bacteria (LAB) especially *Lactobacillus* species are common in milk and milk products (Mitsuoka, 1992).

Probiotic bacteria are mostly delivered in a food system and must be acid and bile tolerant to survive in the human gastrointestinal tract. The time from ingestion to passing through the stomach has been estimated to be approximately 90 minutes (Berada *et al.*, 1991).

Tolerance level to acidic environment of all species of *Lactobacillus* isolated in this study was found to be significantly variable ($p<0.05$) as expressed in table (1). *L. acidophilus, L. casei and L. plantarum* were most resistant at pH 4.0 and their viable count increased. However, at pH 2.0 their viable counts decreased. In contrast *L. bulgaricus* couldn't survive at pH 4.0 and its viable count reached zero at pH 2.0.

The pH, physical and chemical characteristics of a food carrier in which potential probiotics are relayed may have a buffering effect against gut pH, and therefore, positively influence their survival during gastric passage (Charalampopoulos *et al*., 2002; and Patel *et al.*, 2004). This may explain why *L. bulgaricus* known to exhibit poor survival when challenged in vitro to gastric acidity, showed high survival rates in the terminal ileum of fistulated minipigs fed with yoghurt (Lick *et al*., 2001). Comparable results have been reported by Liong and Shah (2005) who indicated that *L. acidophilus* and *L. casei* survived best under acidic conditions. *In vitro* survival of bacterial strains at low pH is a more accurate indication of their ability to survive passage through the stomach.

The organisms taken orally have to face stresses from the host which begin in the stomach, with pH between 1.5 and 3.0 (Corzo and Gilliland, 1999).

Table 1. Mean values (±SE) of plate count (Log10) of isolated lactobacilli at different pH values

pH	*L. acidophilus*	*L. bulgaricus*	*L. casei*	*L. plantarum*
7.0	4.33 ± 0.33^{b}	5.66 ± 0.33^{b}	5.00 ± 0.57^{b}	5.33 ± 0.33^{b}
4.0	6.66 ± 0.33^{c}	1.00 ± 0.57^{a}	6.33 ± 0.88^{b}	5.33 ± 0.88^{b}
2.0	2.73 ± 0.17^{a}	0.00 ± 0.00^{a}	2.00 ± 0.57^{a}	1.33 ± 0.33^{a}

Means having different superscripts in a column or row are significantly different ($P<0.05$).

Antibiotic resistance of the isolated lactobacilli species is summarized in table (2). The four species were sensitive towards most tested antibiotics. Notable observation is the resistance to ciprofloxacin, expressed by all isolates. Antibiotic resistance of microorganisms used as probiotic agents is an area of growing concern. It is believed that antibiotics used for food-producing animals can promote the emergence of antibiotic resistance in bacteria present in the intestinal microflora. Then, these antibiotic-resistant bacteria can transfer resistance factors to other pathogenic bacteria through the exchange of genetic material (Mathur and Singh, 2005). One of the safety considerations in probiotics is verifying that a potential probiotic strain does not contain transferable resistance genes (Temmerman *et al.*, 2003).

Table 2. Antibiotic sensitivity of the lactobacilli species isolated from yoghurt samples

Lactobacillus species	Diameter of inhibition zone in mm					
	AMC	TE	VA	SMZ	CTX	CIP
L. acidophilus	30(S)	0(R)	25(S)	35(S)	27(S)	0(R)
L. bulgaricus	32(S	30(S)	25(S)	32(S)	30(S)	0(R)
L. casei	31(S)	0(R)	25(S)	28(S)	27(S)	0(R)
L. plantarum	30(S)	27(S)	24(S)	29(S)	25(S)	0(R)

Antibiotics (Disk potency): AMC: Amoxiclav (30µg); TE: Tetracycline (30µg); VA: Vancomycin (30µg); SMZ: Sulfamethoxazole (100µg); CTX: Cefotaxime (10 µg); CIP: Ciprofloxacin (5 µg); (S): sensitive; (R): resistant.

The antagonistic effect of the isolated LAB species on some common pathogenic bacteria was evaluated using the agar-spot method. Results in table (3) showed that all LAB species exhibit antagonistic effect on both Gram-positive and Gram-negative bacteria. Statistically, there was no significant difference between the lactobacilli species regarding their antagonistic effect on pathogenic bacteria. However, when this effect was compared with that of commercial probiotics, a significant ($p<0.05$) difference was observed. All lactobacilli isolates exhibited better inhibitory effects than commercial probiotics. The *in vitro* inhibition of the pathogenic isolates by the probiotic species may have been due to the secretion of organic acids, primarily lactic acid, which decreases the pH of the medium to a level unsuitable for growth (Marianelli *et al.*, 2010).

Table 3. *In vitro* antagonistic effect of the *Lactobacillus* sp. on the pathogenic bacteria and the mean (±SE) diameters of zones of inhibition (in mm) in the agar spot assay

Test pathogens	*L. acidophilus*	*L. bulgaricus*	*L. casei*	*L. plantarum*	Commercial Probiotic
Escherichia coli	24.83±0.60[a]	24.00±2.08[a]	27.33±4.05[a]	25.00±0.57[a]	20.00±2.88[b]
	Bactericidal	Bactericidal	Bactericidal	Bactericidal	Bactericidal
Klebsiella pneumoniae	27.33±3.84[a]	27.33±1.76[a]	27.00±1.52[a]	28.00±3.05[a]	20.66±.66[b]
	Bactericidal	Bactericidal	Bactericidal	Bactericidal	Bactericidal
Proteus mirabilis	25.00±2.88[a]	28.33±2.40[a]	28.33±4.40[a]	27.00±1.73[a]	20.66±2.96[b]
	Bactericidal	Bactericidal	Bactericidal	Bactericidal	Bactericidal
Pseudomonas aeruginosa	30.00±2.88[a]	27.00±1.52[a]	27.66±3.71[a]	26.33±0.88[a]	22.66±1.45[b]
	Bactericidal	Bactericidal	Bacteriostatic	Bacteriostatic	Bactericidal
Staphylococcus aureus (MRSA)	25.00±2.88[a]	26.66±2.02[a]	28.00±2.30[a]	25.00±2.88[a]	21.33±0.66[b]
	Bactericidal	Bactericidal	Bacteriostatic	Bactericidal	Bactericidal
Bacillus subtilis	23.33±1.66[a]	27.00±2.51[a]	27.33±1.45[a]	29.00±3.21[a]	23.66±0.66[b]
	Bactericidal	Bactericidal	Bactericidal	Bactericidal	Bactericidal

Means having different superscripts in a column or row are significantly different (P<0.05).

Different reports showed that most lactobacilli strains produce substances that inhibit pathogenic, non-pathogenic and spoilage organisms in fermenting foods and beverages. In general, the antimicrobial activity of lactobacilli may be due to lactic acid, acetic acid, formic acid, phenyllactic acid, caproic acid, organic acids, ethanol organic acids, hydrogen peroxide, bacteriocins or other inhibitory metabolites. Lactic acid and acetic acid are particularly important compounds, inhibiting a broad range of microorganisms (Helander *et al.*, 1997). Yoghurt bacteria are especially effective for the prevention and treatment of some diseases mediated by pathogenic microorganisms through several mechanisms, such as the production of the above mentioned substances (which are active at low pH). In addition, they are able to eliminate the colonization with pathogenic bacteria and treat gastrointestinal tract infections (Petti *et al*., 2008).

It is especially worth noting that most of the antimicrobial activities exhibited by the probiotics were bactericidal in nature, with the exception of *L. casei* which was bacteriostatic against both *Staphylococcus aureus* and *Pseudomonas aeruginosa*. This is consistent with an earlier investigation which showed that the bacterium isolated from yoghurt and identified as *L. casei* was bacteriostatic against methicillin susceptible *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Chuayana *et al.*, 2003).

The viability of the *Lactobacillus* species was investigated during storage at three different temperatures, the results demonstrated that storage at -20°C and 4°C had no effect (p<0.05) on the viable count of all isolates even after 6 weeks of storage as shown in tables 4 and 5. A significant (p<0.05) decline in viable count was observed for all isolates at room temperature after 6 weeks of storage as indicated table 6. Low temperature storage of yoghurt enables use of these LAB species as probiotics. These results agree with that of Ashrad *et al*. (2009).

Table 4. Mean values (M±SE) of viable count (Log10) of isolated lactobacilli during storage at -20°C.

Weeks of storage	*L. acidophilus*	*L. bulgaricus*	*L. casei*	*L. plantarum*
1st	4.63±0.44	4.33±0.33	4.33±0.33	4.50±0.28
2nd	4.46±0.29	4.33±0.33	4.50±0.28	4.50±0.50
3rd	4.66±0.16	4.33±0.33	4.16±0.44	4.33±.88
4th	4.33±0.33	4.00±1.15	4.33±0.33	4.33±0.63
5th	4.33±0.33	4.00±0.57	4.33±0.88	4.50±0.28
6th	4.00±0.57	4.33±0.33	4.66±0.33	4.66±0.33

Table 5. Mean values (M±SE) of viable count (Log10) of isolated lactobacilli during storage at 4°C

Weeks of storage	*L. acidophilus*	*L. bulgaricus*	*L.casei*	*L. Plantarum*
1st	4.63±0.44	4.33±0.33	4.33±0.33	4.50±0.28
2nd	4.46±0.29	4.33±0.33	4.50±0.28	4.50±0.50
3rd	4.66±0.16	4.33±0.33	4.16±0.44	4.33±.88
4th	4.33±0.33	4.00±1.15	4.33±0.33	4.33±0.63
5th	4.33±0.33	4.00±0.57	4.33±0.88	4.50±0.28
6th	4.00±0.57	4.33±0.33	4.66±0.33	4.66±0.33

Table 6. Mean values (M± SE) of viable count (Log10) of isolated lactobacilli during storage at room temperature.

Weeks of storage	*L. acidophilus*	*L. bulgaricus*	*L. casei*	*L. plantarum*
1st	4.80±0.35[b]	4.63±0.08[c]	4.43±0.23[b]	4.66±0.06[d]
2nd	4.56±0.12[b]	4.70±0.05[c]	4.60±0.05[b]	4.66±0.06[d]
3rd	4.46±0.31[b]	4.46±0.24[c]	4.50±0.32[b]	4.03±0.31[c]
4th	4.43±0.29[b]	3.66±0.12[b]	3.60±0.15[a]	3.70±0.15b[c]
5th	3.50±0.28[a]	3.56±0.38[b]	3.50±0.15[a]	3.40±0.11[b]
6th	3.30±0.35[a]	2.73±0.14[a]	2.96±0.41[a]	2.80±0.11[a]

Means having different superscripts in a column or row are significantly different (P<0.05).

4. Conclusion

Development of resistance to antibiotics by bacteria is inevitable, not only because of their high rates of mutation and transferability of drug resistance genes, but also because antibiotics pose a selective pressure against these bacteria, prompting drug-resistant strains to out compete the susceptible ones (Pray, 2008). The rise of multidrug resistant strains has therefore, led researchers to look for alternative therapies such as probiotics that would decrease our reliance on antibiotic use. It is concluded from the present study that species of *Lactobacillus* isolated from homemade yoghurt have the ability to survive at low pH and low temperatures and had strong antagonistic effects against several pathogenic bacteria. These results also suggest that the consumption of products containing probiotics can protect an individual from developing infections caused by pathogenic bacteria. Therefore, probiotics may be helpful in addressing the worldwide issue of antibiotic resistance.

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